## REMARKS

Continued Examination under 37 C.F.R. § 1.114

Applicant acknowledges the Examiners grant of continued examination and further that all objections and rejections not reiterated in the August 13, 2003 office action have been withdrawn.

Claim rejections - 35 U.S.C. § 112.

Claims 83-84 and 98 stand rejected under 35 U.S.C. § 112, first paragraph. With respect to claim 83 and 84 the Examiner states the specification, while being enabling for plants expressing the viral antigens at the levels set forth in the working examples, does not reasonably provide enablement for transgenic plants expressing a recombinant animal viral antigen protein at any level or the levels recited in the claims.

Applicants have amended claims 84 to more clearly define and describe the invention. Claim 84 recites the specific expression level exemplified in the specification. With respect to claim 83, applicant submits that the specification is enabling for achieving the levels of expression necessary to elicit an immune response in an animal upon consumption as exemplified in the examples and the detailed teaching of the specification. Applicants respectfully request Examiner to withdraw this rejection.

Claim 98 was rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art in which it pertains or with which is most nearly connected to make and/use the invention. The Examiner agrees that the specification is enabled for expressing a recombinant TGEV S-protein or a hepatitis B surface antigen protein, but states that it is not enabled for any recombinant protein from any source. Applicant respectfully submits that the specification is enabled for other proteins from other sources. The specification goes into great detail about other antigens that may be useful for practicing the invention. For example at pages 21 and 22, numerous viral antigens are listed which may be used in both humans and animals according to the invention, including: Poliomyelitis, Measles, Mumps, Rubella, Smallpox, Yellow Fever, Viral hepatitis B, Influenza, Rabies, Adenoviral infections, Japanese B encephalitis, Varicella, as well as canine distemper, rabies, canine hepatitis, parvovirus, feline leukemia, Newcastle, Rinderpest, hog cholera, blue tongue and foot-mouth brucellosis, fowl cholera, anthrax and black leg.

Claim rejections under 35 U.S.C. § 112 second paragraph

Claim 88 stands rejected as indefinite for the use of the term "wherein said plant tissue is administered orally". This claim has been canceled.

Claim 98 stands rejected as indefinite for use of the term "expressing a recombinant protein", the Examiner suggests that the claim be amended to indicate that the recombinant protein is a recombinant animal viral antigen protein. The Examiner is thanked for the suggestion and the claim has been so amended.

Claim 99 stands rejected as indefinite for the use of the phrases "which triggers the production of antibodies", and "which is derived from a hepatitis B surface antigen or

transmissible gastroenteritis virus spike protein" and for the use of the term "derived" rather than "obtained". Applicant has amended the claim to remove this language.

Claim 100 stands rejected for the recitation of the phrase "which triggers a mucosal immune response to a viral protein" and for the recitation "plant part or a crude plant extract". This language has ben removed from the claim.

Claim rejections 35 U.S.C. § 102

Claims 73-75, 88, 91 and 98-100 stand rejected under 35 U.S.C. § 102(e) as being anticipated under Goodman et al. (U.S. Patent No. 4, 956, 282). The Examiner states the antigenic property of the expressed protein is considered to be an inherent property of the protein itself. The Examiner asserts that Goodman teaches the expression of animal viral antigen proteins in transgenic plants and that the antigenic properties of the animal viral antigen protein disclosed by Goodman are considered to be inherent to the protein.

Although not conceding to the Examiner's rejection, Applicants have amended all independent claims to clearly encompass features not present or contemplated in the Goodman reference.

The Goodman reference discloses the production of primarily digestive enzymes (See column 5 lines 55-60) in plants and notes that these recombinant enzymes retain physiologic activity when purified from the plant. In other words Goodman states the desire, but does not show experiments or data, that these enzymes maintain their original physiological activities; are properly folded, etc. and remain catalytically active. In the invention here, it is not important or necessary that the protein retain physiologic activity. Instead, the protein structure needs to be maintained long enough to elicit an immune response. Further, in 1988 at the time of the

Goodman reference the state of the art was such when that an antigen expressed in a plant is consumed by an animal it was unknown whether it would be either digested or not presented to the immune system to allow for recognition. Presented herewith for the Examiner's review at Tab A is an article from *Science* September 1994 which discusses the problems encountered with oral vaccines, an excerpt of which follows

> Mucosal vaccine researchers have been stymied before during the decades they've been working on these preparations, and many of the same obstacles remain. Despite early successes with live attenuated oral vaccines against tuberculosis and polio more than 30 years ago, the expected heyday for mucosal vaccines never followed. One problem was that the chemical attenuation methods then in use altered the genes and pathogens randomly, in many cases changing them enough so that they failed to trigger a vigorous immune response. Other mucosal vaccines, which are usually ingested orally, inhaled, or taken as nose drops, fell victim to destruction by bodily defenses such as enzymes and acids in the stomach, problems not faced by serum vaccines.. . .
>
> Service, Robert F., Research News, _Science_ 263:1522-1524 "Triggering the First Line of Defense" (1994).

Thus, art-recognized problems associated with oral vaccines are not taken into account in Goodman. Critical to achieving vaccine activity is expression of the antigen at levels necessary to provide for an immune response. The levels of expression disclosed in Goodman would not be high enough to achieve the response contemplate by applicant's invention.

Further, Applicants have been recognized by peers as the first real (or enabling) teaching of production of immunogenic viral proteins in plants. Provided with this amendment at Tab B is a copy of Gomez et al, "Expression of Immunogenic Glycoprotein S Polypeptides from Transmissible Gastroenteritis Coronavirus in Transgenic Plants" _Virology_ 249:352-358 (1998). In this article, the authors cite inventor, Mason's 1992 description of the present invention as the first introduction of vaccine production in transgenic plants. (see p.352, col. 2 citing Mason et al, "Expression of hepatitis B surface antigen in transgenic plants" _Proc. Natl. Acad._ Sci 89:11745-

11749 (1992), copy also attached at Tab B). They discuss the Mason descriptions and follow its teachings to express TGEV in *Arabidopsis.* Thus, this author recognizes the present invention as the first to teach TGEV expression in plants and successfully uses those teachings to later express the TGEV.

Goodman does not disclose either inherently or directly, anything about controlling the levels of expression of this enzyme to achieve a) a quantity necessary in a plant so that an immune response is obtained upon oral consumption, b) expression directed to edible tissues or portions of the plant, or c) expression in monocots. Each of the independent claims has been amended to include recitation of one of these features of applicant's invention that is nowhere present in Goodman.

Support for these amendments is found on pages 8 line 12, and page 23 lines 20-25 and page 24 line 5 which define and gives examples of edible plants useful for the invention; page 9 lines 18-25 and page 26 lines 8-25 which disclose tissue specific promoters that can be used to preferentially direct expression to the edible portions of a plant; page 16 and page 23 lines 16-19 which disclose monocots, and page 34 which discusses the use of leader sequences and enhancers. All of these features are nowhere present in Goodman and are critical to establishing applicants invention which is the production in a plant of not simply a protein that remains physiologically active when purified, but production in a plant of a viral antigen so that the plant itself when consumed presents a viral epitope to the immune system so that the animal elicits an immune response to that protein.

Applicants respectfully request that Examiner withdraw this rejection.

Conclusion

Applicants thank the Examiner for the Interview and in the event that it is felt that it would aid prosecution, she is invited to contact the undersigned at the number listed to discuss this case. Reconsideration and allowance is respectfully requested.

This is a request under the provision of 37 CFR § 1.136(a) to extend the period for filing a response in the above-identified application for one month from November 13, 2003 to December 13, 2003. Applicant is a small entity; therefore, please charge Deposit Account Number 26-0084 in the amount of $55.00 for one month to cover the cost of the extension. Any deficiency or overpayment should be charged or credited to Deposit Account 26-0084.

Respectfully submitted,

Heidi Nebel, Registration No. 37,719
McKEE, VOORHEES & SEASE, P.L.C.
801 Grand Avenue, Suite 3200
Des Moines, Iowa 50309-2721
Phone No. (515) 288-3667
Fax No. (515) 288-1338
**CUSTOMER NO: 22885**

Attorneys of Record

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# Triggering the First Line of Defense

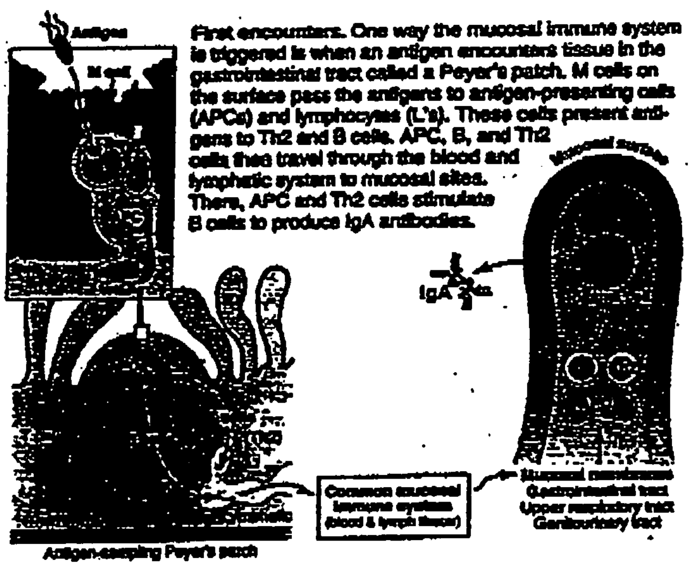
Vaccines that activate mucosal immunity, often the body's first chance to ward off infection, have been hard to come by. That situation is beginning to change. And these new vaccines are needle-free

For decades, vaccine researchers have been fighting infectious diseases without much help from one of the body's major defensive weapons. Most successful vaccines to date, such as the childhood measles, mumps, and rubella immunizations, have been made from injected antigens that spark the body to produce blood-circulating, or serum, antibodies against disease-causing organisms. But the part of the immune system which churns out 70% of the body's antibodies has been virtually ignored, because little was known about how it works.

That part is the mucosal immune system. Membranes covered with mucous (a microorganism-trapping gel) line the airways, the reproductive system, and the gastrointestinal (GI) tract, and many pathogens, such as the bacterium that causes cholera and the virus that causes AIDS, first encounter the body there. "The mucosal surface is the first port of entry of many diseases," says George Lowell, a vaccinologist at the Walter Reed Army Institute of Research in Washington, D.C., who is trying to develop mucosal vaccines. "The hope is that [mucosal vaccines] can stop the virus or bacteria before it gets in."

Now, it seems, researchers are figuring out ways to bring this weapon to bear. As immunologists gain knowledge about the mucosal system, vaccines aimed at eliciting mucosal immunity are coming on line. Those furthest along are typically made from live organisms that have been genetically altered, or attenuated, to insure that they are no longer capable of causing disease. One such anti-cholera preparation recently went on the market in Switzerland and is currently under review in several other European countries. A similar live vaccine for typhoid was approved for use in the United States in 1989. Other live mucosal vaccines are being developed to fight everything from the flu to rabies.

And live preparations are not the only strategies being used. Developers are also cloaking mucosal antibody-triggering antigens in protective coatings to improve their delivery rate or packaging them alongside powerful chemicals called adjuvants which boost antibody responses (see table on p. 1524). "This area is exploding," says mucosal vaccine developer Jerry McGhee, who directs the Immunobiology Vaccine Center at the University of Alabama at Birmingham (UAB).




**First encounters.** One way the mucosal immune system is triggered is when an antigen encounters tissue in the gastrointestinal tract called a Peyer's patch. M cells on the surface pass the antigens to antigen-presenting cells (APCs) and lymphocytes (L's). These cells present antigens to Th2 and B cells. APC, B, and Th2 cells then travel through the blood and lymphatic system to mucosal sites. There, APC and Th2 cells stimulate B cells to produce IgA antibodies.

ILLUSTRATION: K. SUTLIFF

Creating an immunological first line of defense is just one reason for the boom. Another is ease of delivery. Researchers are developing many mucosal vaccines to be delivered orally, rather than injected, and oral vaccines represent the Holy Grail to many health experts, says Harvard University microbiologist and vaccine developer John Mekalanos. The United Nations' 1992 Children's Vaccine Initiative calls for oral vaccines for at least 15 diseases, because they are easy to deliver and do away with the need for needles, which can transmit diseases such as AIDS and hepatitis. If mucosal vaccines reach their potential, "it would be the best protection we have," says Marian Neutra, a cell biologist at Harvard who investigates how the mucosal immune system confers protection.

That "if," however, is a substantial one. Mucosal vaccine researchers have been stymied before during the decades they've been working on these preparations, and many of the same obstacles remain. Despite early successes with live attenuated oral vaccines against tuberculosis and polio more than 30 years ago, the expected heyday for mucosal vaccines never followed. One problem was that the chemical attenuation methods then in use altered the genes of pathogens randomly, in many cases changing them enough so that they failed to trigger a vigorous immune response. Other mucosal vaccines, which are usually ingested orally, inhaled, or taken as nose drops, fell victim to destruction by bodily defenses such as enzymes and acids in the stomach, problems not faced by serum vaccines. Finally, in some cases the protection conferred by mucosal vaccines proved fleeting, fading after only a few months or a year.

Such obstacles brought down several mucosal vaccines, including those developed in early attempts to combat cholera and dysentery. And although the base of knowledge about the mucosal immune system has grown, researchers today are still contending with such problems. As a result, most vaccine researchers continue to develop serum vaccines, says Neutra. "It would be nice to have simpler means for administering vaccines," says Mary Lou Clements, who heads the Johns Hopkins Center for Immunization Research. "But mucosal vaccines still need to overcome some hurdles."

### Another form of protection

Most researchers' hopes for leaping those barriers lie in their growing understanding of how different immune responses are produced. While serum immune responses involve antibodies of the class known as immunoglobulin G, or IgG, which travel through the blood, immune cells in mucosal surfaces primarily churn out a different type of antibody, IgA, which is released along mucosal surfaces and helps pick invaders off before they can gain entry.

Immunologists have known for some time that the key to producing IgA appears to lie in small clumps of tissue in the GI tract, the nasal and respiratory passages, and other mucosal surfaces that contain various white

blood cells—lymphocytes—responsible for generating specific immunity. In the GI tract, for example, groups of lymphoid cells known as Peyer's patches serve as "quality-control inspectors," sampling bits of food proteins and microorganisms as they pass through the small intestine. Immune cells known as M cells that sit on the surface of the Peyer's patches engulf the small particles and pass them along to a collection of other immune cells lying in the interior of the Peyer's patch: antigen-presenting cells (APCs) and cells called T and B cells. The particles are broken down further by the APCs, which present selected bits on their surface to the B cells, which are the IgA antibody factories (see diagram on p. 1522).

While mucosal immunity typically involves the production of IgA and its release into the mucosal surfaces, antigens picked up by mucosal lymphoid tissues can also trigger the production of blood-circulating IgG antibodies. *Vibrio cholerae*, the cholera bacterium, for example, typically triggers both serum and mucosal antibodies. So in measuring whether a vaccine is effective, what researchers look for is not whether specific IgA levels have risen, but whether a vaccine offers protection against a later challenge by the pathogen.

## Going live

To trigger these responses, researchers have begun to design antigen carriers that are specifically targeted to Peyer's patches and other lymphoid tissues that sample antigens. For example, *V. cholerae* is in many ways an ideal carrier, as it naturally colonizes the GI tract and is readily picked up by M cells. The bug's own defenses also make it adept at avoiding destruction by enzymes or stomach acids. Of course, the organism does cause disease, so researchers who want to use it as a protectant need to short-circuit its virulence.

They do so by removing some toxic genes—and it can take a while to figure out how best to do that. In the case of the live attenuated cholera vaccine unveiled in Europe, for instance, University of Maryland (UM) microbiologist James Kaper began work in 1981 to remove the genes from the organism that code for diarrhea-causing toxins, leaving untouched others, such as surface proteins that help trigger an immune response. The advantage of this approach, says UM vaccinologist Myron Levine, who led the team that developed the vaccine, is that "there is much less likelihood that an organism will revert back to a virulent strain by undergoing a random mutation."

It wasn't until the mid-1980s that the UM group was able to produce a strain that was harmless, yet still able to trigger a protective immune response. Now, however, the strategy seems to be working. In trials involving nearly 200 healthy American volunteers, Levine's group showed that just one dose of vaccine provided 100% protection against the moderate and severe diarrhea that is the hallmark of the disease. These studies were not designed to measure whether the vaccine provided lasting protection; that question, says Levine, should be answered by long-term field trials now underway in 67,000 people in Indonesia.

Researchers are also using live attenuated vaccines to try to trigger mucosal immunity to other afflictions. Several groups, including Levine's group at the University of Maryland and Brian Murphy at the National Institute of Allergy and Infectious Diseases in Bethesda, Maryland, are attempting to attenuate pathogens to fight typhoid fever and influenza, as well as shigellosis, a major diarrheal disease in developing countries.

## Organisms as shuttles

Not every pathogen, of course, conveniently heads straight for M cells when it enters the body. So researchers have begun to introduce DNA snippets from such organisms into the genome of another that is better suited to trigger a mucosal response. The ability of organisms such as *V. cholerae* and *Salmonella typhi* to preferentially bind to M cells makes them ideal carriers. Several groups, including those headed by Levine and by McGhee, are experimenting with *S. typhi* to express proteins from the bugs that cause malaria, diphtheria, whooping cough, and tetanus. They have had preliminary success in getting *S. typhi* to express the new proteins, and animal studies have shown that vaccines made with a similar bug that infects animals can confer protection against challenges.

Another carrier generating interest is

**Vaccines needed.** About 20,000 people perished in Rwanda's cholera epidemic this summer. A cholera vaccine that triggered mucosal immunity would have been effective prevention.

Bacille Calmette-Guérin, or BCG, a bacterium originally used as an oral vaccine against tuberculosis. (An injected anti-TB vaccine is currently most used in this country, as the oral vaccine is sometimes difficult to administer to young children.) At MedImmune, a biotechnology firm in Gaithersburg, Maryland, researchers are using recombinant BCG as a delivery vehicle for proteins to fight Lyme disease, urinary tract infections, and tuberculosis. Earlier this year, MedImmune's Solomon Langermann showed that when he delivered recombinant BCG-expressing antigens from *Borrelia burgdorferi*—the organism responsible for Lyme disease—intranasally to mice, all of the animals received protection against subsequent challenges by the organism.

These results are particularly striking, Langermann says, because the vaccine triggers not just a mucosal IgA response, but also serum IgG antibodies. Such blood-circulating antibodies are likely to be essential to the success of a Lyme disease vaccine in humans, says Langermann, because the organism makes its way directly into the blood through tick bites. Another plus is that the protection offered by the vaccine seems to last. Langermann's ongoing studies have shown no drop in antibody levels 18 months after administration. That may be due in part to the influence of BCG, Langermann hypothesizes: The BCG vaccine against tuberculosis provides lifetime protection. "But the intranasal route may actually be responsible for inducing long-lasting antibody-forming cells in the circulation," he adds. In any case, says Langermann, "I'm hopeful that it will be a good vector against a variety of mucosal pathogens."

One problem with the use of live organisms to deliver antigens from other pathogens is that the patient may develop antibodies against the delivery vehicle. And those antibodies will cause a reaction against that organism if the body meets it again. As a result, most vehicles can be used only once. Such "vector immunity" is a problem for researchers working with *V. cholerae*, says Levine, as there are only two strains, or serogroups, of the organism. This means such organisms will have limited use as shuttles, because a vaccine for one disease could spoil the field for later vaccines that also use *V. cholerae*. Other organisms, like *S. typhi*, contain more than 2000 such serogroups, giving researchers plenty of live delivery vehicles to choose from.

## Molecular shields

Another way researchers are attempting to shuttle antigens past host defenses such as stomach acids is to encapsulate them in tiny microspheres. The most widely used microspheres are made from a biodegradable polymer called poly (DL-lactide-co-glycolide), or PLG. In the mid-1980s, John Eldridge, then

at UAB and now with Lederle–Praxis Biologicals in West Henrietta, New York, first showed that PLG microspheres could shroud antigens and deliver them to immune cells in the respiratory and GI tracts and generate an immune response. Now researchers are using PLG microspheres to deliver antigens against everything from influenza to diarrhea-causing *Escherichia coli*.

At OraVax in Cambridge, Massachusetts, for example, vaccinologist Tom Monath is experimenting with PLG microspheres to deliver antigens against enterotoxigenic *E. coli*, or ETEC, a principle cause of diarrhea in developing countries. For their anti-ETEC antigen, Monath and his colleagues purified the tiny hairlike cilia that house a "colonization factor" used by ETEC to bind to epithelial cells in the gut. When Monath's collaborators at the Walter Reed Army Institute of Research and UM gave the oral vaccine to healthy volunteers in 1992, they found that two of 10 volunteers were immunized against ETEC. While far from perfect, the fact that encapsulating antigens was effective at all is "quite encouraging," says Monath, who is now preparing for a new round of trials designed to boost the dose of the vaccine in hopes of increasing the percentage of volunteers protected.

In addition to PLG, researchers are experimenting with making microspheres out of other materials, such as liposomes, which are made from the same materials that compose cellular membranes (*Science*, 15 July, p. 316). Many researchers see antigen encapsulation as having a particularly bright future, because the vehicles are being made from common biological materials and typically don't trigger a vector immune response. "I think they are the way of the future, quite honestly," says Mekalanos, who is developing his own live attenuated vaccine against cholera.

Yet, like other delivery systems, antigen capsules have drawbacks. "The missing piece of the puzzle is efficiency," says Mekalanos, explaining that only a few percent of the capsules are taken up by lymphoid cells such as M cells. That's a problem for microsphere vaccines, as in general the less antigen, the less antibody produced. Live vaccines usually sidestep this problem, because they reproduce naturally, supplying ample amounts of antigen.

To boost the uptake of microspheres, researchers control the size of the capsules to between 1 and 10 microns, which for as-yet-unknown reasons are preferentially taken up by M cells. Some groups are also working to target the microspheres more directly. At UAB, for example, Suzanne Michalek is attempting to coat the outer surface of PLG microspheres with the receptor-binding portions of the cholera toxin, which has a special affinity for binding to M cells.

In addition to boosting uptake, researchers are attempting to make the antigens they deliver trigger a stronger immune response by adding chemicals known as adjuvants that heighten the immune response to antigens they accompany. UAB's Ray Jackson reported last year that when mice were given cholera toxin along with antigens from the organism that causes tetanus, all were subsequently protected from a tetanus challenge; without cholera toxin the mice received no protection.

**Making protection last**

Although progress has been made in many of these areas, one hurdle that's proven particularly stubborn for most mucosal vaccines, says Clements, is the need to maintain permanent protection. This clearly isn't impossible in principle. The examples of the oral tuberculosis and polio vaccines show that the mucosal immune system is capable of generating the "memory cells" that remain ready to trigger an immune response if the same invader is encountered later on.

But in some cases, the mucosal immune system doesn't seem to provide this effective memory-based resistance, as certain pathogens, such as respiratory syncytial virus and rotavirus, can reinfect children again and again. "It suggests that mucosal immunity doesn't provide solid protection" against all infections, says Clements. And that may mean researchers will have better luck generating lasting protection against some diseases than against others. Perhaps vaccines that trigger more than one type of immune response, such as recombinant BCG, will help researchers achieve long-lasting protection. But to become commercially viable, most vaccines will have to generate immunity for longer than the year and a half achieved by such vaccines to date. Even if long-lasting protection is not achieved, some vaccines, such as Levine's cholera vaccine, may be appropriate as short-term protection for travelers.

And although they do away with needles, mucosal vaccines face their own set of practical difficulties. Oral vaccines are difficult to give to infants, because babies don't swallow on command or may spit up. And if they are still breast-feeding, antibodies in the mother's milk can pick off potential vaccines before they can produce an immune response. "These are all things that have to be looked at," says Clements.

But in spite of these obstacles, there's increasing interest in the search for effective mucosal immunity. "I think there is going to be an explosion of knowledge in the next 6 years on delivering vaccines without needles," says Barry Bloom, an immunologist at the Albert Einstein College of Medicine in New York City. And many in the field hope that the effects of the blast, and the protection of the vaccines, last for a long time.

–Robert F. Service

**SOME MUCOSAL VACCINES UNDER DEVELOPMENT**

| Disease | Pathogen | Vaccine Vector |
|---|---|---|
| Cholera | *Vibrio cholerae* | Live attenuated and [illegible] bacteria |
| Diarrhea | Enterotoxigenic *Escherichia coli* | Microspheres, live attenuated bacteria |
| | Rotavirus | Microspheres, live attenuated virus |
| | *Clostridium difficile* | Live recomb. *Shigella* |
| | *Shigella* species | Microspheres, live attenuated bacteria |
| Typhoid fever | *Salmonella typhi* | Live attenuated bacteria |
| Upper respiratory tract infection | Parainfluenza virus | Microspheres, live attenuated virus |
| [illegible] | Influenza virus | Microspheres, live attenuated virus |
| Pneumonia | Respiratory syncytial virus | Microspheres, live attenuated virus |
| | *Streptococcus pneumoniae* | Live recomb. *Salmonella* |
| Lyme disease | *Borrelia burgdorferi* | Live recomb. BCG |
| AIDS | HIV | Live recomb. polio virus and *Salmonella*; microspheres, adjuvants |
| Tooth decay | *Streptococcus mutans* | Microspheres, adjuvants |
| Meningitis | *Plasmodium falciparum* | Live recomb. *S. typhi* |
| Sepsis, skin infections | *Staphylococcus aureus* | [illegible] |
| Rabies | Rabies virus | Live recomb. adenovirus |
| Mononucleosis, [illegible] cancer in AIDS patients | Epstein-Barr virus | Live recomb. adenovirus |

Pat Sweeney
1835 Pleasant St., W st Des Moines, IA 50265
Phon : (515)222-0921 Fax: (515)267-0556

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VIROLOGY 249, 352–358 (1998)
ARTICLE NO. VY989315

# Expression of Immunogenic Glycoprotein S Polypeptides from Transmissible Gastroenteritis Coronavirus in Transgenic Plants

N. Gómez,* C. Carrillo,† J. Salinas,‡ F. Parra,§ M. V. Borca,† and J. M. Escribano*[1]

*Centro de Investigación en Sanidad Animal (CISA-INIA), Valdeolmos, 28130 Madrid, Spain; †Instituto de Virología, CICV, INTA Castelar, Buenos Aires, Argentina; ‡Departamento de Biotecnología y Mejora Genética, INIA, 28040 Madrid, Spain; and §Instituto Universitario de Biotecnología de Asturias, Facultad de Medicina, Universidad de Oviedo, 33006 Oviedo, Spain

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The use of transgenic plants as vaccine production systems was described recently. We report on the immunological response elicited by two recombinant versions of the glycoprotein S from the swine-transmissible gastroenteritis coronavirus (TGEV) expressed in transgenic plants. Arabidopsis plants were genetically transformed with cDNAs constructs encoding either the N-terminal domain (amino acid residues 1–750) or the full-length glycoprotein S of TGEV, responsible for the neutralizing antibody induction against the virus, under the control of the cauliflower mosaic virus 35S (CaMV 35S) promoter. Genomic DNA and mRNA analyses of leaf extracts from transformed plants demonstrated the incorporation of the foreign cDNA into the arabidopsis genome, as well as their transcription. Expression of recombinant polypeptides were observed in most transgenic plants by ELISA using specific antibodies. Mice immunized with leaf extracts from transgenic plants developed antibodies that reacted specifically with TGEV in ELISA, immunoprecipitated the virus-induced protein, and neutralized the virus infectivity. From those results, we conclude that transgenic plants expressing glycoprotein S polypeptides may possibly be used as a source of recombinant antigen for vaccine production. © 1998 Academic Press

## INTRODUCTION

Swine-transmissible gastroenteritis virus (TGEV) is the causative agent of acute diarrhea of newborn piglets that provokes high mortality rates in affected farms. Protective immunity against this disease must be developed in pregnant sows to confer passive protection to the piglets through colostrum and milk. Neutralizing antibodies against the virus are directed mainly to glycoprotein S (Garwes et al., 1978; Jimenez et al., 1986), and relevant epitopes in neutralization have been mapped into the N-terminal domain of this protein (Correa et al., 1988). Four major antigenic sites have been described in glycoprotein S, of which site A is the immunodominant (De Diego et al., 1992; Delmas et al., 1990; Sánchez et al., 1990). Glycoprotein S from TGEV has been expressed using different vectors with tropism that favored antigenic presentation in the mucosal surfaces (Smerdou et al., 1996; Torres et al., 1995). These vaccination approaches promoted systemic and mucosal antibody induction and, in the case of adenovirus vector, conferred protection to suckling piglets (Torres et al., 1996).

The development of genetic transformation technology in plants has made possible the expression of foreign genes in different plant species, making reasonable the idea of using plants as bioreactors to produce recombinant proteins. The concept of vaccine production in transgenic plants was first introduced by Mason et al. in 1992. Proteins involved in protective immune response can be produced at a low cost and easily purified from plant extracts for parental inoculation. In addition, oral immunization by edible vaccines produced in transgenic plants could stimulate immune responses at the portal entrance of many pathogens, facilitating the desi[illegible] of large-scale immunization programs. The presen[illegible] of specific antigens into plants, even at low levels, can raise by the oral route immune reactions comparable to those raised by conventional vaccines (Haq et al., 1995; Mason et al., 1996).

Hepatitis B surface antigen (Thanavala et al., 1995), *Escherichia coli* heat-labile enterotoxin (LT-B) antigen (Haq et al., 1995; Tacket et al., 1998), Norwalk virus capsid protein (Mason et al., 1996), VP1 antigen fr[illegible] foot and mouth disease virus (Carrillo et al., 1998), an[illegible] cholera toxin B subunit (Arakawa et al., 1998) are the vaccine antigens expressed in transgenic plants and tested for the immune response elicited in immunized animals. Additionally, rabies virus glycoprotein was expressed in transgenic tomatoes, but the immune response induced by administration of these plants to animals was not tested (McGarvey et al., 1995). In the present study, we investigated the feasibility of expressing the glycoprotein S from TGEV in transgenic plants, as well as th[illegible] antigenicity and immunogenicity of the plant-derived protein. The S protein is an excellent model for developing oral

[1] To whom reprint requests should be addressed. E-mail: escriban@inia.es.

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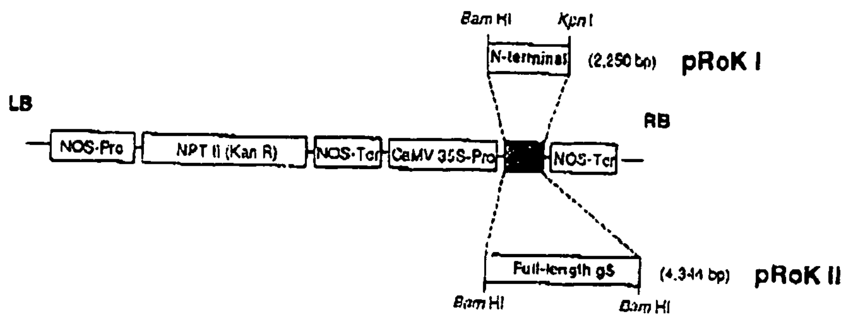


FIG. 1. Schematic structure of the binary plasmids pRoK I and II used for *Agrobacterium*-mediated plant transformation. The DNA sequences encoding for the full-length or N-terminal domain of glycoprotein S from TGEV are cloned downstream of the CaMV 35S promoter in recombinant pRoK plasmids, followed by the nopaline synthase (NOS) terminator. These plasmids contain the left (LB) and right (RB) borders of transferred DNA that demarcate the sequences that are incorporated into the plant genome.

vaccines against enteric pathogens of mammals because of its immunogenicity and resistance to degradation in the gut.

## RESULTS

### Plasmid construction and selection of transgenic plants

The binary pRoK I and pRoK II recombinant plasmids (Fig. 1), carrying a cDNA coding for the N-terminal region or the full-length glycoprotein S respectively, were obtained by subcloning the corresponding sequences from previously obtained constructs. Recombinant pRoK plasmids allow selection of transformants on media containing kanamycin and stable integration into nuclear chromosomal DNA from the plant. pRok uses the cauliflower mosaic virus 35S (CaMW 35S) promoter for nominally constitutive transcription of the cloned genes.

Plant transformation with pRoK I and II was carried out as described in Materials and Methods by *Agrobacterium tumefaciens*-mediated transformation. The transgenic plants resistant to the selective medium appeared similar in morphology to the nontransgenic arabidopsis plants. More than 20 different lines of transformants containing each construct were obtained and self-pollinated to obtain F2 lines. All lines were positive when screened for the presence of the recombinant genes by polymerase chain reaction (PCR) analysis (Fig. 2A).

Most plants harboring recombinant genes showed specific transcription of foreign genes by reverse transcription (RT)-PCR analysis (Fig. 2B). To rule out the possibility of amplification of contaminant DNA sequences present in the RNA preparations, we treated the purified RNA with ribonuclease before foreign gene amplification by using *Taq* polymerase. No amplified DNA fragments were detectable under those conditions, assessing the RNA dependence of the reaction (Fig. 2B).

### Recombinant protein expression in transgenic plants

The presence of the recombinant polypeptides in the plants harboring and expressing the foreign genes was investigated in four plants of each construct, selected to be analyzed by ELISA and Western blotting using an anti-TGEV polyclonal serum. Results demonstrated that leaf extracts from all selected plants were positive on ELISA (Fig. 3). However, no specific reaction on Western blotting was detected in any of the plant extracts analyzed (data not shown), probably due to the low levels of recombinant protein expression and to the conformational nature of most of the immunodominant epitopes present in this protein.

From a titration ELISA using different virus dilutions and a monospecific anti-glycoprotein S antibody, we found that ~30–60 µg of soluble leaf protein contains a glycoprotein S antigenic mass equivalent to that contained in 0.02 µg of purified TGEV. The percentage of the total soluble protein corresponding to recombinant glycoprotein S polypeptides accumulated in the leaves of arabidopsis transformants could represent 0.06–0.03% of the total soluble leaf protein.

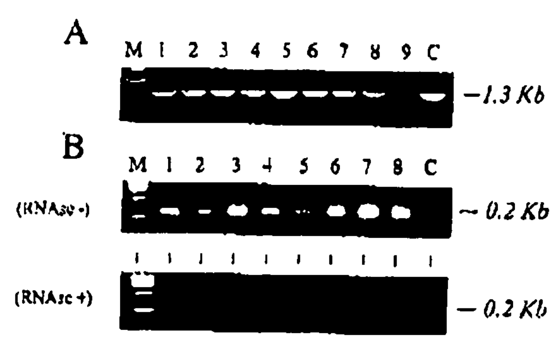


FIG. 2. Characterization of transgenic plants transformed with pRoK I (1–4), pRoK II (5–8), or pRoK 2 (9) plasmids. (A) Presence of the recombinant genes in representative transgenic arabidopsis plants detected by PCR. C, control amplification of the same DNA fragment from pRoK II. (B) Foreign gene transcription in representative transformed plants analyzed by RT-PCR. Samples were treated or not with RNase to assess the DNA specificity of the reactions. C, same analyses in arabidopsis plants transformed with pRoK 2 plasmid.

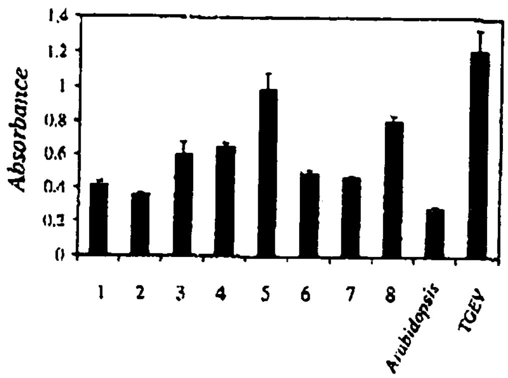


FIG. 3. Detection of N-terminal (1–4) and full-length (5–8) glycoprotein S polypeptides in protein extracts from transgenic plant leaves by ELISA. The figure shows the mean ± SE of the absorbance readings obtained in three independent assays. Positive control is purified virus (TGEV); and negative control is a plant transformed with pRoK 2 plasmid (*Arabidopsis*).

### Antibody induction by plant-derived recombinant proteins

Leaf extracts from transgenic plants expressing the N-terminal (plants 1–4) or full-length glycoprotein S (plants 5–8) were used to immunize mice. A control mouse was immunized with a leaf extract from a plant transformed with pRok2 plasmid. After three immunization doses, the specificity of mice sera was tested by an ELISA using purified TGEV as antigen. Figure 4A shows that all sera reacted with the virus showing, as expected, different titers. A kinetic of antibody induction in an immunized mouse (number 3) was studied by immunoprecipitation of glycoprotein S induced by TGEV in infected ST cells. This mouse serum immunoprecipitated specifically the virus protein after two immunizations (Fig. 4B).

Finally, sera from all immunized mice were tested in a TGEV neutralization assay. Both glycoprotein S polypeptides produced in transgenic plants elicited virus-neutralizing antibodies (neutralization indexes of 2.2–3.5; Fig. 4C). Serum from a nonimmunized mouse (not shown) or from the mouse immunized with the plant transformed with pRoK 2 plasmid did not show virus neutralization activity (Fig. 4C).

## DISCUSSION

In this report, we show that full-length or the globular part (N-terminal domain) of TGEV spike protein (glycoprotein S) expressed in transgenic plants retained the antigenic properties and elicited neutralizing antibodies when used to immunize animals. Expression in eukaryotic hosts is required for antigenic determinants that are dependent on glycosylation. Of the three major antigenic sites defined on glycoprotein S involved in the induction of TGEV-neutralizing antibodies, sites A and B are complex, conformational, and glycosylation dependent. Site D can be represented by synthetic peptides, although glycosylation has a minor effect on its conformation (Gebauer *et al.*, 1991). Several genetically engineered vaccines using prokaryotic vectors have failed against TGEV. Glycoprotein S expressed at high levels in *Escherichia coli* and used to inoculate animals did not induce neutralizing antibodies or confer protection *in vivo* (Hu *et al.*, 1987).

Plant cells present differences in protein glycosylation with respect to animal cells that could determine the lose of antigenic determinants in antigens expressed in transgenic plants. Glycosylation in plants may differ in the extent of glycosylation, processing, or both of N-linked oligosaccharide side chains (Faye *et al.*, 1993). Furthermore, the complex glycans of plants are often smaller than those of animals, in part due to the absence of sialic acid (Faye *et al.*, 1993). The only precedent of a glycoprotein expressed in plants for vaccine development is the glycoprotein G of rabies virus (McGarvey *et al.*, 1995). This protein expressed in tomato plants showed a molecular mass ~4 and ~6 kDa less than that obtained from virus-infected cells but still larger than the protein size predicted for the unglycosylated polypeptide chain (McGarvey *et al.*, 1995). The molecular mass of glycoprotein S expressed in *Arabidopsis thaliana* could not be determined because we were not able to detect the recombinant protein on Western blotting. However, antigenic determinants with strong dependence of glycosylation seem to be preserved because the plant-derived antigens induced neutralizing antibodies in immunized animals, indicating that critical antigenic sites are at least in part correctly glycosylated in plants.

This work demonstrates the feasibility of expressing glycoprotein S polypeptides in plants. Because the site of insertion of the transferred DNA into the cellular chromosomal DNA is random, different levels of protein expression in independent transformants are expected. We obtained expression levels similar to that described with equivalent constructs expressing hepatitis B surface antigen or rabies virus glycoprotein (Mason *et al.*, 1992; McGarvey *et al.*, 1995). More recently, expression levels of Norwalk virus capsid protein in tobacco have been shown to be higher than the above mentioned antigens (up to 0.23% of total soluble protein; Mason *et al.*, 1996). We have not found significant differences in foreign antigen plant expression between the two forms of glycoprotein S studied. The use of different promoters, the use of plant-derived leader sequences and signal peptides, and mainly the modification of the codon usage of this protein could improve expression levels in plants.

The demonstration that many proteins from pathogens, including some expressed in transgenic plants (Haq *et al.*, 1995; Mason *et al.*, 1996), are immunogenic when administered orally, encourages the study of other antigens expressed in plants to develop edible vaccines.

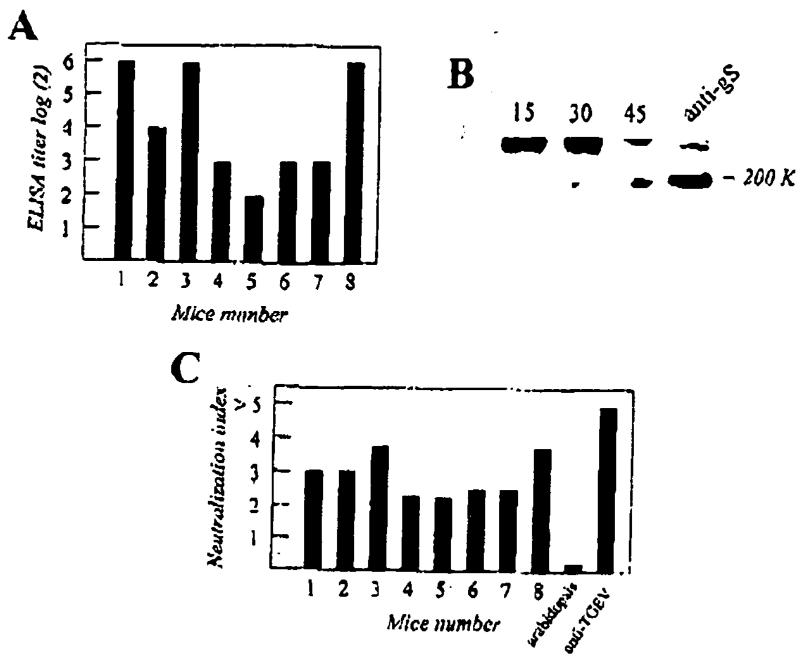


FIG. 4. Antibody responses to the plant-derived glycoprotein S polypeptides in parenterally inoculated mice. (A) ELISA titers of sera from mice inoculated with plant extracts expressing the N-terminal (mice 1–4) or the full-length glycoprotein S (mice 5–8). Titers are referred to the ELISA values obtained with the serum from a mouse immunized with a pRoK 2 transformed plant extract. (B) Kinetic of antibody induction against glycoprotein S in a mouse (mouse 3) after one, two, and three immunization doses (15, 30, and 45 days postinoculation, respectively) analyzed by immunoprecipitation of the glycoprotein S induced by TGEV in infected cells. An anti-glycoprotein S (anti-gS) serum was used as immunoprecipitation control. (C) Neutralization indexes of sera from immunized mice with plant extracts expressing the N-terminal (mice 1–4) or full-length glycoprotein S (mice 4–8). The neutralization index is defined as the ratio between the log of virus titer in the presence of a control mouse serum and sera from mice immunized with transgenic plants expressing the antigens (1–8) or transformed with pRoK 2 (*Arabidopsis*). A rabbit anti-TGEV serum (anti-TGEV) showing high neutralization titer was also used as positive control. ELISA and neutralization index values are the mean of three independent experiments.

Glycoprotein S from TGEV is an interesting model because this protein is resistant, at least when incorporated into the viral particle, to gut degradation. In addition, the protective immune responses against TGEV have to be stimulated at the mucosal surfaces to induce secretory and lactogenic immunity (De Diego *et al.*, 1992, 1993; Saif and Bohl, 1979; Wesley *et al.*, 1988). Once we have determined the feasibility of expressing immunological active polypeptides from TEGV glycoprotein S in plants, studies on the immune response of plant-derived glycoprotein S polypeptides in pigs are necessary.

## MATERIALS AND METHODS

### Plant material and growth conditions

Seeds of *Arabidopsis thaliana* (Heynh, ecotype Columbia) were sown in pots containing a mixture of universal substrate and vermiculite (3:1). To synchronize germination, pots were placed at 4°C for 48 h in darkness and then transferred to a growth chamber at 20°C with a 16-h photoperiod. Irrigation was carried out with distilled water and, occasionally, with a mineral nutrient solution (Haughn *et al.*, 1986).

### Production of transgenic *Arabidopsis*

A 2250-pb cDNA fragment (nucleotides 1–2250; fragment I) and a 4344-pb cDNA fragment (nucleotides 1–4344; fragment II) encoding for the N-terminal and full-length glycoprotein S from TGEV Purdue strain, respectively, were amplified by RT-PCR from viral RNA and cloned into pBacPAK9 plasmid (Clontech). The RT primers used were 5′-CCCAACTATGGTACCATCAAT AACAGC-3′ (complementary primer to nucleotides 2225–2250) and 5′-CGCGGGATCCTTAATGGACGTG-CACTTTTTC-3′ (complementary primer to nucleotides 4313–4344). Then, the cDNA was synthesized by using the primer 5′-GCGCGGATCCATGAAAAACTATTTGT-GG-3′. Subsequently, DNA fragments I and II were subcloned in the binary pRoK2 plasmid (Baulcombe *et al.*, 1985) under the control of the CaMW 35S promoter, yielding the recombinant plasmids pRoK I and pRoK II, respectively (Fig. 1).

Plasmids pRoK I and pRoK II were used for arabidopsis plant transformation as described elsewhere (Bechtold *et al.*, 1993) with slight modifications. *A. tumefaciens* (C58C1 strain) containing pRoK I or pRoK II plasmids was grown in 600 ml of LB medium con-

taining 50 $\mu$g/ml kanamycin until an $OD_{600}$ value of 2 was reached. After centrifugation, bacteria were resuspended in 200 ml of 2.36 g/l Murashige and Skoog medium containing 10 g/l 6-benzilaminopurine and 5% sucrose. The 6–7-week-old plants were immersed in the *A. tumefaciens* suspension by inversion of the pots, and vacuum infiltration was performed in a vacuum chamber at 50 mb for 15 min. Infiltrated plants were rinsed with water and placed in the greenhouse until attaining maturity. Transgenic T1 seeds were selected by germination in Petri dishes containing GM [4.7 g/l Murashige and Skoog, 1% sucrose, 0.5 g/l 2-(*N*-morpholino)ethanesulfonic acid (MES), 8 g/l agar, pH 5.7] and 50 $\mu$g/ml kanamycin. Two-week-old transgenic plants were transplanted into soil and allowed to attain maturity. The plants were self-pollinated to obtain T2 plants and used for further analysis.

## PCR and RT-PCR analyses

The presence of the foreign cDNA sequences in generated transgenic arabidopsis was detected by PCR. Plant extracts were prepared by macerating leaves (~10 mg) with pestle and mortar in 300 $\mu$l of a buffer containing 200 mM Tris–HCl, pH 8.6, 250 mM NaCl, 25 mM EDTA, and 0.5% SDS. The resulting extract was mixed with 150 $\mu$l of 3 M $CH_3COONa$, pH 5.2, and incubated for 10 min at −20°C. Then, samples were centrifuged, and the DNA contained in the supernatant was precipitated and resuspended in 30 $\mu$l of TE buffer. PCR was performed on 0.5 $\mu$g of DNA with a pair of primers that specifically amplify a 1389-bp fragment of the glycoprotein S gene (sense primer, 5′-GCGCGGATCCATGAAAAACTATTTGTGG-3′; antisense primer, 5′-GCGCGGTACCCGATGTGAAGCTATTG-3′).

Glycoprotein S mRNA in transgenic plants was analyzed by RT-PCR. Total RNA from the leaves of transformed plants was isolated using the Fast RNA kit (BIO 101) according to the manufacturer's instructions. RNA was treated with 10 units of DNase–RNase free (RQ1; Promega) during 15 min at 37°C. Then, 1 $\mu$g of total RNA was diluted in 4 $\mu$l of RT buffer [250 mM Tris–HCl, pH 8.3, 15 mM $MgCl_2$, 375 mM KCl, and 10 mM dithiothreitol (DTT)] containing 0.5 mM concentration of each dNTP (Pharmacia). 10 units of RNasin (RNase inhibitor, human placenta; Boehringer-Mannheim), 200 units of reverse transcriptase (Moloney murine leukemia virus RT; GIBCO BRL), and 100 pM concentration of the antisense primer (5′-GCGCGGTACCAAACCAAGGTTGTACAG-3′) in a final volume of 20 $\mu$l. The mixture was incubated for 1 h at 37°C. Denaturation of RNA–cDNA hybrids and inactivation of the reverse transcriptase were done by boiling the reaction for 5 min. To the RT mixture, we added 50 pM concentration of the above mentioned sense primer and 5 units of *Taq* DNA polymerase (GIBCO BRL). This reaction specifically amplifies a 197-bp fragment of the glycoprotein S gene. Treatments of purified RNA samples with RNase (Promega) were carried out with 10 units of the enzyme for 15 min at 20°C.

## Detection of glycoprotein S in transgenic plants

Proteins from leaves were obtained by homogenization of leaves in a blender with liquid nitrogen, and the resulting powder was resuspended in buffer (0.3 g of fresh wt/ml) containing 10 mM MES, pH 6, 10 mM NaCl, 5 mM EDTA, 0.6% Triton X-100, 0.25 M sucrose, 0.15 mM spermine, 0.5 mM spermidine, 10 mM DTT, and 1 mM phenylmethylsulfonyl fluoride. The extract was filtered and centrifuged 10 min at 12,000 *g*, and the resulting supernatant was used for glycoprotein S polypeptides expression analyses.

ELISA plates were coated with 100 $\mu$l (10 $\mu$g/ml of PBS) of a mixture of two monoclonal antibodies, 6AC3 and 8DH8 (kindly provided by Dr. L. Enjuanes, Centro Nacional de Biotechnologia, CSIC, Spain), recognizing the antigenic sites A and D of the glycoprotein S, respectively (Correa *et al.*, 1988). Antibodies were incubated for 12 h at 4°C, and then plates were washed and blocked 1 h at 37°C with 5% fetal bovine serum in PBS containing 0.05% Tween 20. After washing the plates, leaf proteins from transgenic plants (15 $\mu$g of total soluble protein per well, diluted in 200 $\mu$l of PBS, pH 7), containing full-length or the N-terminal domain of glycoprotein S, were added to react with the previously adsorbed antibodies in the microtiter ELISA plates during 12 h at 4°C. Plates were then washed six times with 0.05% Tween 20 in PBS, and 100 $\mu$l of rabbit anti-S protein, obtained after three immunization doses with the baculovirus-expressed N-terminal fragment of glycoprotein S and diluted at 1:100 in PBS containing 0.05% Tween 20, was added per well and left to react for 1 h at 37°C. Plates were washed again six times with PBS–Tween 20 buffer, and immunocomplexes were incubated with Protein A-peroxidase (Sigma) diluted 1:1000 in PBS–Tween 20 for 1 h at 37°C. Finally, plates were washed again, and 200 $\mu$l of a freshly prepared solution of *o*-phenylenediamine dihydrochloride (Sigma) and $H_2O_2$ was added. Reactions were stopped with 2 N $H_2SO_4$, and the absorbance was measured at 492 nm.

## Induction of anti-glycoprotein S antibodies

BALB/c mice (one per arabidopsis plant) were immunized intramuscularly on days 0, 15, and 30 with leaf extract in PBS (40 $\mu$g of total protein per animal per injection) in complete Freund's adjuvant for the first inoculation and in incomplete adjuvant for the others.

Mice sera were evaluated for anti-glycoprotein S-specific antibodies by ELISA using purified TGEV as antigen. Coated ELISA plates with 100 $\mu$l of PBS, pH 7.4, containing 0.2 $\mu$g of virus were blocked as described above with

5% fetal bovine serum, and after washing of the plates six times, sera diluted 1:10 in PBS–Tween 20 were added (100 μl per well) and incubated for 1 h at 37°C. Then, plates were washed again to remove unbound antibodies, and goat anti-mouse antibodies (1:500) were added to reveal immunocomplexes. After being washed and developed with o-phenylenediamine dihydrochloride substrate as described above, reaction was stopped with 2 N $H_2SO_4$, and plates were read at 492 nm.

Immunoprecipitation of glycoprotein S by sera from a mouse after different immunization doses was carried out essentially as previously described for mouse antibodies (Bullido *et al.*, 1996). Briefly, ST cells infected with TGEV (m.o.i. 5) were incubated for 14 h, pulse labeled for 1 h with 200 μCi/ml of $^{35}$S-methionine (800 Ci/mmol; Amersham International, Amersham, England)/ml, and lysed with lysis buffer (10 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, pH 7.4, 1 mg/ml bovine serum albumin, and 1 mM phenylmethylsulfonyl fluoride). The lysate ($10^6$ cpm) was incubated with a control mouse serum (15 μl) for 1 h and precleared with a 25% (v/v) suspension of Protein G–Sepharose (Pharmacia, Sweden) in lysis buffer. The precleared $^{35}$S-labeled cell extract was incubated with mice sera (15 μl) for 2 h at 4°C, and immunocomplexes were incubated with 25% suspension of Protein G–Sepharose for 1 h with gentle mixing. Beads were washed three times with lysis buffer and boiled in SDS–electrophoresis buffer. The antigen–antibody complexes were analyzed in 7.5% SDS–PAGE.

A plaque reduction assay with sera from immunized mice was performed as described previously (Jiménez *et al.*, 1986). The neutralization index of each serum was expressed as the $\log_{10}$ of the ratio of the pfu/ml of virus obtained using a normal serum and that observed in the presence of a given anti-glycoprotein S mouse serum.

## ACKNOWLEDGMENTS

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Immunology

# Expression of hepatitis B surface antigen in transgenic plants

(oral vaccine/foreign genes/plants)

HUGH S. MASON*†, DOMINIC MAN-KIT LAM*‡, AND CHARLES J. ARNTZEN†§

*AgriStar Inc., 100 Hawthorn, Conroe, TX 77301; †Institute of Biosciences and Technology, Center for Plant Biotechnology, Texas A&M University, Houston, TX 77030-3303; and ‡LifeTech Industries, Ltd., 100 Hawthorn, Conroe, TX 77301

*Contributed by Charles J. Arntzen, September 16, 1992*

**ABSTRACT** Tobacco plants were genetically transformed with the gene encoding hepatitis B surface antigen (HBsAg) linked to a nominally constitutive promoter. Enzyme-linked immunoassays using a monoclonal antibody directed against human serum-derived HBsAg revealed the presence of HBsAg in extracts of transformed leaves at levels that correlated with mRNA abundance. This suggests that there were no major inherent limitations of transcription or translation of this foreign gene in plants. Recombinant HBsAg was purified from transgenic plants by immunoaffinity chromatography and examined by electron microscopy. Spherical particles with an average diameter of 22 nm were observed in negatively stained preparations. Sedimentation of transgenic plant extracts in sucrose and cesium chloride density gradients showed that the recombinant HBsAg and human serum-derived HBsAg had similar physical properties. Because the HBsAg produced in transgenic plants is antigenically and physically similar to the HBsAg particles derived from human serum and recombinant yeast, which are used as vaccines, we conclude that transgenic plants hold promise as low-cost vaccine production systems.

Hepatitis B virus infection is one of the most widespread viral infections of humans and causes acute and chronic hepatitis and hepatocellular carcinoma (1). The infectious viral particle (Dane particle) is a 43-nm double-shelled sphere that consists of a core containing the 3.2-kilobase (kb) DNA genome bound to the core protein, surrounded by the viral envelope containing phospholipids and the major surface antigen [hepatitis B surface antigen (HBsAg)] (2). In addition to Dane particles, the serum of infected individuals also contains 22-nm subviral particles in great excess over virions. These noninfectious particles contain the elements of the viral envelope, including the major 24-kDa peptide that occurs in glycosylated and unglycosylated forms (2).

Because the host range of hepatitis B virus is limited to humans and chimpanzees, and since the virus cannot be propagated in cell culture, HBsAg for use in vaccines was purified from the serum of infected individuals until a recombinant form (rHBsAg) was produced in yeast (3). The immunogenic yeast-derived rHBsAg occurs in the form of spherical particles with an average diameter of 17 nm. Integration of the peptides into the phospholipid-containing particles greatly enhances their immunogenic properties (4). Subsequent work showed that the peptides present in the yeast-derived particles were much less extensively disulfide-linked than in the human material but that such linking could be induced *in vitro* (5).

Intramuscular injection of serum-derived HBsAg or yeast-derived rHBsAg in healthy individuals results in effective immunization and protection from viral infection (6, 7). In many areas of the developing world, however, the expense of immunization programs for large segments of the population is prohibitive. This has led us to attempt the expression of rHBsAg in plants with the hope of developing a less expensive production system. Further, we hope to find a way to present the rHBsAg in edible plant tissues in a form that would be useful as an oral vaccine. In this paper we describe the transformation of tobacco with the gene encoding HBsAg and its expression in leaf tissue in the form of an antigenic spherical particle with an average diameter of 22 nm. This plant-derived rHBsAg is directly analogous to the rHBsAg from yeast that is now used for commercial vaccines. We view this as a successful first step in a long-term project dedicated to developing technologies for low cost "edible vaccines" for the developing world.

## MATERIALS AND METHODS

**Construction of Plasmids for Plant Transformation.** The HBsAg coding region on the *Pst* I/*Hin*dIII fragment from pMT-SA (kindly provided by Li-he Guo, Chinese Academy of Sciences) was subcloned into pBluescript KS (Stratagene) to form pKS-HBS. The HBsAg gene in pKS-HBS was opened 116 base pairs (bp) 3′ to the termination codon with *Bst*BI and the resulting ends were blunted by filling with Klenow enzyme and dCTP/dGTP. The entire coding region was then excised 16 bp upstream of the *Pst* I site with *Bam*HI. pBI121 (ref. 8; obtained from Clontech) was digested with *Sac* I and the ends were blunted with mung bean nuclease. The GUS coding region was then released with *Bam*HI and the vector was isolated. The HBsAg coding fragment was ligated into the GUS-less pBI121 to yield pHB101 (Fig. 1), where its expression is driven by the cauliflower mosaic virus (CaMV) promoter derived from pBI121.

The CaMV 35S promoter with duplicated enhancer linked to the tobacco etch virus (TEV) 5′ nontranslated leader sequence, which acts as a translational enhancer (9), was excised from pRTL2-GUS (10) as follows. pRTL2-GUS was digested with *Nco* I and the ends were blunted with mung bean nuclease. The promoter-leader fragment was then released by digestion with *Hin*dIII. pHB101 was digested with *Hin*dIII and *Sma* I to release the 35S promoter fragment, and the vector was purified. The promoter-leader fragment was then ligated into the *Hin*dIII/*Sma* I-digested pHB101 to yield pHB102 (Fig. 1). The HBsAg coding region lies upstream of the nopaline synthase terminator in both constructs. The plasmids contain the left and right border regions, which denote the limits of the DNA that is integrated into the plant genomic DNA via *Agrobacterium tumefaciens*-mediated transformation, as well as the neomycin phosphotransferase gene, which allows selection with kanamycin.

**Plant Transformation.** *Agrobacterium* strain LBA4404 cells were transformed by the direct method (11) with the plasmids prepared from *Escherichia coli* clones, and the

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Abbreviations: CaMV, cauliflower mosaic virus; HBsAg, hepatitis B surface antigen; rHBsAg, recombinant HBsAg; TEV, tobacco etch virus.

§To whom reprint requests should be addressed.

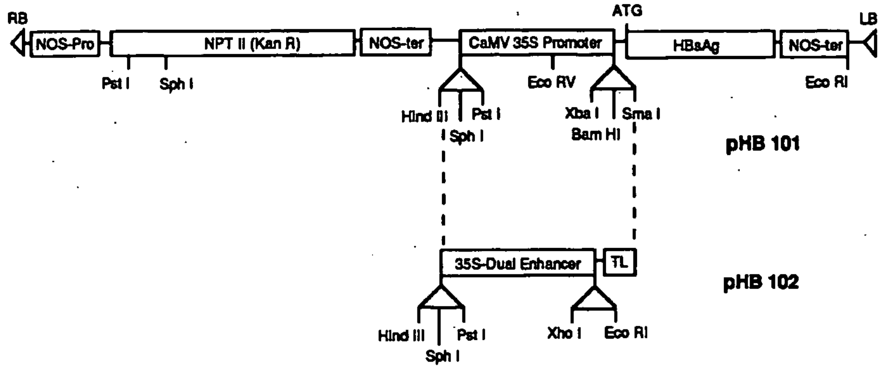


FIG. 1. Structure of plasmids pHB101 and pHB102. The constructs carry the left and right borders (LB, RB) of the transferred DNA that demarcates the sequences that are incorporated into the plant genomic DNA via *Agrobacterium*-mediated transformation. The HBsAg coding region lies downstream of the CaMV 35S promoter in pHB101 and is followed by the nopaline synthase (NOS) terminator. In pHB102, the 35S promoter is replaced by a modified CaMV 35S promoter containing a duplicated transcriptional enhancer region, linked to the TEV 5' nontranslated leader (TL). Restriction endonuclease cleavage sites are indicated. Blunt-ended ligation has removed the *Nco* I site at the 3' end of the leader sequence.

structure of the plasmids was verified by restriction digestion. Tobacco (*Nicotiana tabacum* cv. Samsun) was transformed by cocultivating leaf discs (12) with *Agrobacterium* strains transformed with pHB101 or pHB102. Shoots were generated from transformed callus selected on medium containing 0.2 mg of kanamycin per ml and 0.2 mg of cefotaxime per ml. Shoots were rooted in medium containing 0.1 mg of kanamycin per ml, transplanted to soil, and watered with one-half strength Hoagland medium.

**Analysis of RNA from Transformed Tobacco.** Total RNA from leaves of plants transformed with pHB101 was isolated as described (13). The RNA was denatured with formaldehyde, fractionated on 1% agarose gels (5 $\mu$g per lane), blotted to nylon, and probed with $^{32}$P-labeled random-primed DNA using a 700-bp *Bam*HI/*Acc* I fragment from pKS-HBS that includes most of the coding region for HBsAg. Blots were hybridized at 68°C in 0.25 M sodium phosphate, pH 7.0/1 mM EDTA/7% SDS, washed with 40 mM sodium phosphate, pH 7.0/5% SDS at 68°C, and exposed to X-Omat AR film for 4 hr.

**Analysis of Protein from Transformed Tobacco.** Protein was extracted from leaf tissues by homogenization with a Ten-Broek ground glass homogenizer (clearance, 0.15 mm) in 5 volumes of buffer containing 20 mM sodium phosphate (pH 7.0), 0.15 M NaCl, 20 mM sodium ascorbate, 0.1% Triton X-100, and 0.5 mM phenylmethylsulfonyl fluoride at 4°C. The homogenate was centrifuged at 1000 × *g* for 5 min, and the supernatant was centrifuged at 27,000 × *g* for 15 min. The 27,000 × *g* supernatant was centrifuged at 100,000 × *g* for 1 hr, and the pellet was resuspended in extraction buffer. Protein in the different fractions was measured by the Coomassie dye-binding assay (Bio-Rad). HBsAg was assayed with the Auszyme monoclonal kit (Abbott), using the positive control (HBsAg derived from human serum) as a standard. The positive control was diluted to give HBsAg levels of 0.09–1.8 ng per assay, and the absorbance at 492 nm after color development gave a linear relationship in this range.

**Immunoaffinity Purification of HBsAg from Transgenic Tobacco.** Monoclonal antibody against HBsAg (clone ZMHB1) was obtained from Zymed Laboratories. The immunogen source for this antibody is human serum. The antibody was bound to Affi-Gel Hz hydrazide gel (Bio-Rad) according to the instructions supplied with the kit. Soluble material that was resuspended from the 100,000 × *g* pellet was made to 0.5 M NaCl and mixed with the immobilized antibody-gel by end-over-end mixing for 16 hr at 4°C. The gel was washed with 10 volumes of 10 mM sodium phosphate, pH 7.0/0.5 M NaCl, and 10 volumes of 10 mM sodium phosphate, pH 7.0/0.15 M NaCl, and bound HBsAg was eluted with 0.2 M glycine (pH 2.5). The eluate was immediately neutralized with Tris base, and particles were pelleted at 109,000 × *g* for 1.5 hr at 5°C. The pelleted material was negatively stained with phosphotungstic acid and visualized with transmission electron microscopy using a Phillips CM10 microscope.

**Sucrose and CsCl Gradient Analysis of HBsAg from Transgenic Tobacco.** Extracts of leaf tissues were made as described above and 0.5 ml of the 27,000 × *g* supernatants was layered on linear 11-ml 5–30% sucrose gradients made in 10 mM sodium phosphate, pH 7.0/0.15 M NaCl or discontinuous 12-ml CsCl gradients (1.1–1.4 g/ml) made in 10 mM sodium phosphate at pH 7.0 (3 ml each of 1.1, 1.2, 1.3, and 1.4 g of CsCl per ml). Positive control HBsAg from the Auszyme kit was also layered on separate gradients. The sucrose gradients were centrifuged in a Beckman SW41Ti

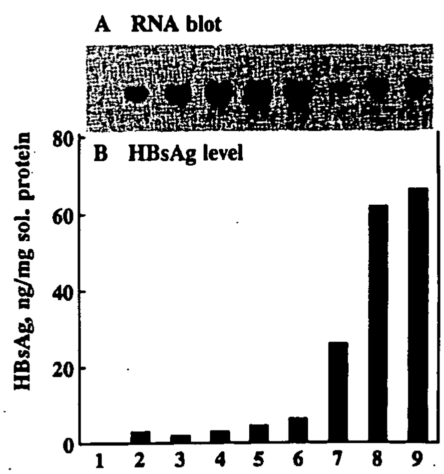


FIG. 2. HBsAg mRNA and protein levels in transgenic tobacco plants. (*A*) Total RNA from wild-type untransformed or independent transgenic tobacco lines carrying either the pHB101 or the pHB102 construct was hybridized with a probe specific for the HBsAg coding region. (*B*) Protein extracts from the same leaves were tested for HBsAg with the Auszyme monoclonal kit (Abbott), and HBsAg levels were quantified using a standard curve of human serum-derived HBsAg. Numbers: 1, wild-type control plant; 2–6, independent transformants harboring the construct in pHB101; 7–9, independent transformants harboring the construct in pHB102.

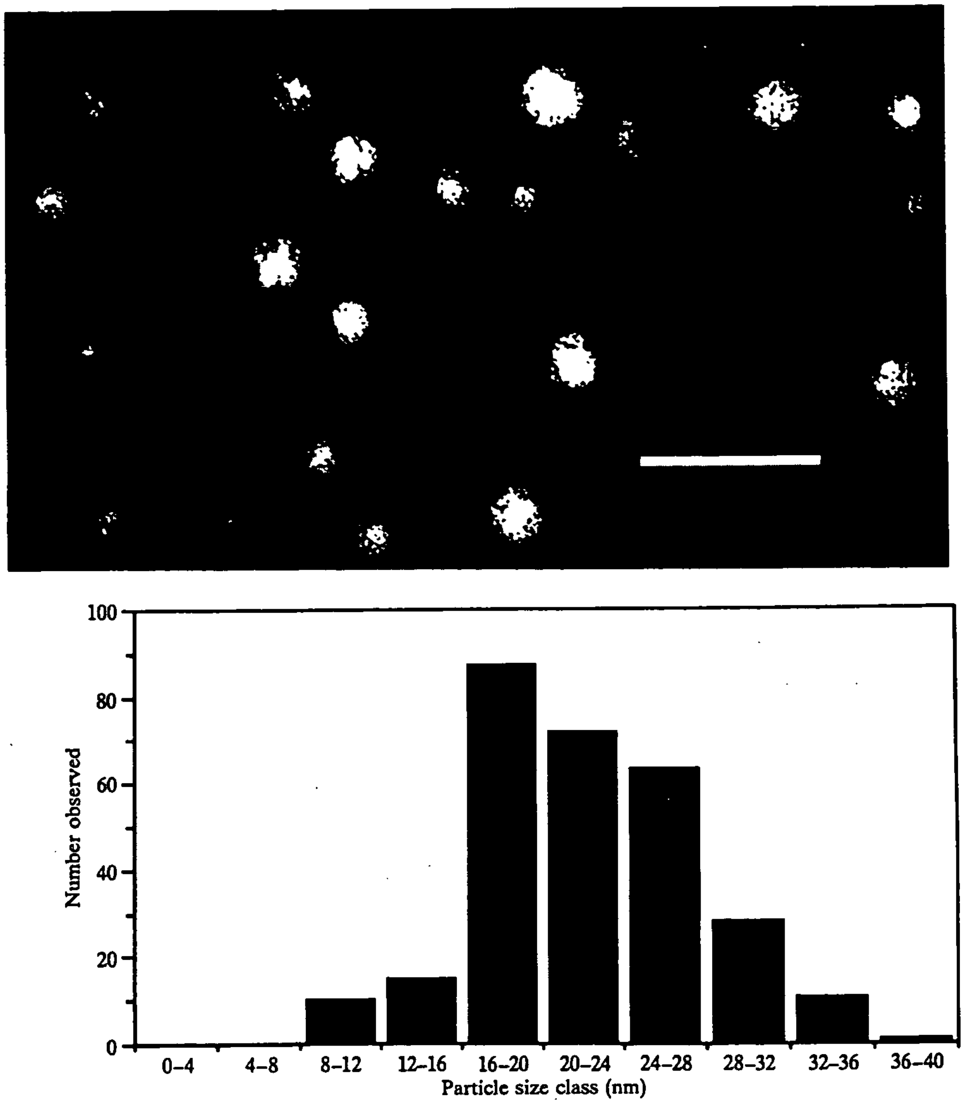


FIG. 3. (*Upper*) Electron micrograph of immunoaffinity purified rHBsAg. HBsAg from a transgenic tobacco plant harboring construct pHB102 was purified by immunoaffinity chromatography, negatively stained with phosphotungstic acid, and visualized by transmission electron microscopy. (Bar = 100 nm.) (*Lower*) Histogram generated by measuring the diameters of the particles observed in the representative field.

rotor at 33,000 rpm for 5 hr at 5°C and fractionated into 1-ml fractions while monitoring the $A_{280}$. The CsCl gradients were centrifuged in a Beckman SW40Ti rotor at 30,000 rpm for 25 hr at 5°C and fractionated into 1.0-ml fractions. HBsAg in the gradient fractions was assayed using the Auszyme kit as described above. The density of gradient fractions was measured by weighing aliquots with an analytical balance.

## RESULTS AND DISCUSSION

The plasmid HB101 (Fig. 1) was constructed by inserting the coding region for HBsAg from pMT-SA between the *Bam*HI and *Sac* I sites in the plant transformation vector pBI121 after removal of the GUS coding region. In this construct the expression of HBsAg is driven by the CaMV 35S promoter. A modification of pHB101 was made by inserting the CaMV 35S promoter with dual transcriptional enhancer linked to the TEV 5′ nontranslated leader (10) in the place of the original 35S promoter to form pHB102 (Fig. 1). The TEV leader acts as a translational enhancer to increase the amount of protein made using a given amount of template mRNA (9).

The plasmids were used to transform tobacco by the leaf disc method using *Agrobacterium*, and regenerated kanamycin-resistant transformants were analyzed by hybridizing RNA samples with a labeled probe encompassing the coding region of the HBsAg gene. Fig. 2*A* shows the results of an experiment where selected transformants harboring either the pHB101 or the pHB102 construct and a wild-type control were probed. The signals were variable between transformants, as expected due to effects of position of insertion into the genomic DNA and differing copy number. The transcripts from the pHB101 transformants (Fig. 2*A*, lanes 2–6) were ≈1.2 kb in length by comparison with RNA standards (H.S.M., unpublished data), which is consistent with the expected size. The pHB102 transcripts were slightly larger (Fig. 2*A*, lanes 7–9), owing to the 5′ addition of the TEV leader sequence. The nontransformed control leaf RNA (Fig. 2*A*, lane 1) showed no detectable signal at this stringency of hybridization. Thus, mRNA that hybridizes specifically with HBsAg probe was present in the leaves of selected transformants, and there is no inherent transcriptional limitation to the expression of HBsAg in tobacco leaves.

Using the HBsAg assay kit, we tested leaf extracts for the presence of material that reacts specifically with monoclonal antibody to serum-derived HBsAg. Fairly low levels were observed for the pHB101 transformants, ranging from 2 to 6 ng/mg of soluble protein (Fig. 2*B*, nos. 2–6). The pHB102 transformants showed substantially greater levels of HBsAg, ranging up to 66 ng/mg of soluble protein (Fig. 2*B*, nos. 7–9). The reaction was specific because wild-type tobacco showed no detectable HBsAg (Fig. 2*B*, no. 1). The levels of HBsAg observed in the individual transformants were roughly proportional to the levels of specific mRNA encoding HBsAg for a given construct. The pHB102 transformants, containing the 5′ TEV leader, showed much higher accumulations of HBsAg for a given amount of mRNA than did the pHB101 transformants (Fig. 2). The translational enhancement observed in mRNAs carrying the TEV leader appears to involve a cap-independent competition for translation initiation factors (9).

HBsAg from human serum occurs as ≈22-nm spherical particles, consisting of protein embedded in a phospholipid bilayer. Since rHBsAg from plasmid-transformed yeast also occurs as particles of a similar size class, we sought evidence that the recombinant material in tobacco is present as particles. We observed that 95% of the HBsAg in the 27,000 × *g* supernatants of transgenic tobacco leaf extracts pelleted at 200,000 × *g* for 30 min (H.S.M., unpublished data), suggesting a particle form. We purified HBsAg by immunoaffinity chromatography using a monoclonal antibody raised against human serum-derived HBsAg. Inspection of this material by negative staining and transmission electron microscopy revealed the presence of particles ranging in diameter between 10 and 40 nm (Fig. 3). Most of the particles were between 16 and 28 nm (Fig. 3 *Lower*); the average diameter was 22 nm. These are very similar to the particles observed in human serum (2), although we observed no rods. The rHBsAg particles from yeast occur in a range of sizes with a mean of 17 nm (3). We conclude that the rHBsAg made in transgenic tobacco retains the capacity for self-association and thus has the physical properties of human serum-derived HBsAg and rHBsAg from yeast, both of which are highly immunogenic in the particle form.

We obtained further evidence of particle behavior from sedimentation and buoyant density studies of transgenic tobacco leaf extracts. Fig. 4 shows a sucrose gradient profile of HBsAg activity from transgenic tobacco harboring the construct in pHB102. The plant-derived HBsAg sedimented with a peak near the 60S ribosomal subunit, and the serum-derived material sedimented in a somewhat sharper peak just slightly slower. These data are consistent with the finding that human HBsAg sediments at 55 S (14). The observation that the plant material sedimented slightly faster and with a broader peak than the human HBsAg is also consistent with the larger mean size of the plant particles and wider range of sizes (Fig. 3). The buoyant density of the rHBsAg from transgenic tobacco in CsCl was found to be ≈1.16 g/ml (Fig. 5), whereas the human particle showed a density of about 1.20 g/ml. Thus, the rHBsAg from transgenic tobacco exhibits sedimentation and density properties that are very similar to the subviral particles obtained from human serum. Importantly, HBsAg in the particle form is found to be much more immunogenic than that in the form of the peptide alone (4).

The subcellular localization of the HBsAg in our transgenic plants has not been characterized. Particles are observed in the lumen of the endoplasmic reticulum in infected liver cells (15) and appear to be secreted by the constitutive secretory pathway. The peptides contain two signal sequences, one N-terminal and one internal, that together determine a transmembrane orientation of the molecules (2). Whether the rHBsAg particles in leaf cells are secreted to the extracellular space or retained within the cytoplasm is a question that needs to be addressed. Although the HBsAg coding region is part of a larger open reading frame in the viral genome (2), the lack of the pre-S peptide does not alter the formation of particles in transfected mammalian cells (16) or yeast (3). Our construct also contains no pre-S sequences and is thus similar to that used in those studies. We were unable to analyze the size of the HBsAg peptides produced in transgenic tobacco because the monoclonal antibodies that we used failed to recognize the SDS-denatured peptides on SDS/PAGE blots. The antibodies did recognize undenatured HBsAg in dot blots of leaf extracts or blots of whole leaves or seedlings, however (H.S.M., unpublished data).

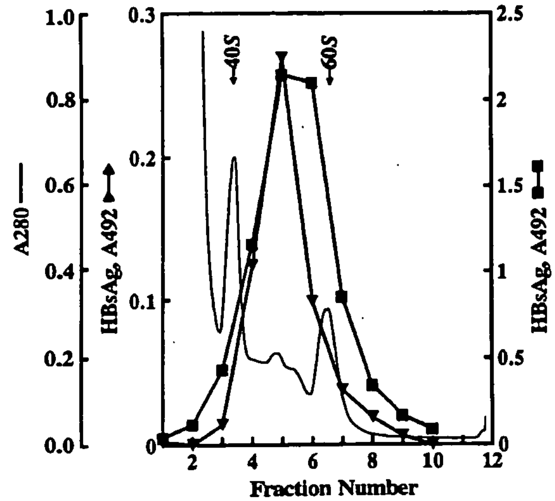


FIG. 4. Sucrose density gradient sedimentation of HBsAg from transgenic tobacco. Soluble fractions from a transformant harboring the pHB102 construct or human serum-derived HBsAg were sedimented in 5–30% sucrose gradients, fractionated, and assayed for HBsAg. The solid curve represents the absorbance profile at 280 nm of the plant extract. The top of the gradient is at the left; positions of the 40S and 60S ribosomal subunits are indicated. ■, HBsAg in transgenic plant extract; ▼, HBsAg in serum-derived material; solid line, absorbance of tobacco leaf extract at 280 nm.

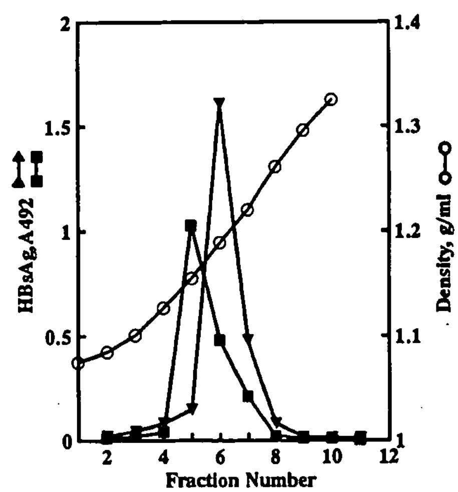


FIG. 5. Buoyant density in CsCl of HBsAg from transgenic tobacco. Supernatant fractions of transgenic tobacco harboring construct pHB102 and human serum-derived HBsAg were banded in a 1.1–1.4 g/ml CsCl gradient. Fractions were assayed for HBsAg activity. ■, HBsAg in plant extract; ▼, HBsAg in serum-derived material; ○, density of gradient fractions.

In conclusion, we have shown that HBsAg can be expressed in plant tissues via stable transformation with foreign DNA. Furthermore, the rHBsAg from transgenic tobacco is recognized specifically by monoclonal antibodies directed against human serum-derived HBsAg and is processed properly after translation so that the antigenic particle form is observed. These studies indicate the feasibility of expression of foreign antigens in plants for possible use as oral vaccines. Presently, the maximal levels of HBsAg we have found in transgenic plants represent ≈0.01% of the soluble leaf protein. This is an inadequate level for the efficient use of plants as production systems for rHBsAg for vaccine use. Further studies must be done to increase the accumulation of HBsAg, such as using other transcriptional regulatory elements to increase mRNA levels. The processing of the HBsAg peptides in plant tissues must also be examined, specifically with regard to glycosylation and intermolecular disulfide bonding. The HBsAg system may be useful for determining the feasibility of targeting foreign antigens to specific subcellular compartments.

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